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Award Number: W81XWH-12-1-0248

TITLE: Targeted Inhibition of Tyrosine Kinase-Mediated Epigenetic Alterations to Prevent Resurgence of Castration-Resistant Prostate Cancer

PRINCIPAL INVESTIGATOR: Dr. Kiran Mahajan

CONTRACTING ORGANIZATION: H. Lee Moffitt Cancer Center

REPORT DATE: October 2013

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE October 2013	2. REPORT TYPE Grant Award Final	3. DATES COVERED 2 July 2012 - 1 July 2013

4. TITLE AND SUBTITLE Targeted Inhibition of Tyrosine Kinase-Mediated Epigenetic Alterations to Prevent Resurgence of Castration-Resistant Prostate Cancer

5a. CONTRACT NUMBER
W81XWH-12-1-0248

5b. GRANT NUMBER
W81XWH-12-1-0248

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S) Dr. Kiran Mahajan and Dr. John Koomen

E-Mail: kiran.mahajan@moffitt.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Moffitt Cancer Center, Tampa Fl 33612

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Epigenetic alterations leading to the silencing of tumor suppressor genes or activation of oncogenes are underlying causes for metastatic disease. The proposed project set out to identify and quantify novel epigenetic modifications- histone phosphorylations -mediated by oncogenic kinases, that may promote the progression of prostate cancer to androgen independence by using differentially labeled prostate cancer cell lines grown in the presence and absence of androgen. SILAC based mass spectrometry analysis revealed that lysine residues in histones were found to be acetylated at a number of sites and threonine and serine residues were phosphorylated in both androgen dependent and castration resistant prostate cancer cells. Notably, a significant decrease in acetylation of lysine 5 in histone H2B, was observed in androgen deprived cells. Although tyrosine phosphorylation was detected by immunoblotting of histones prepared from prostate cancer cells, specific tyrosine residues were not uncovered by LC-MS/MS analysis. ACK1 and WEE1 tyrosine kinase signaling were found to be some of the major pathways upregulated in prostate cancer cells upon androgen deprivation. Future studies could explore H2BK5Ac, WEE1, and ACK1 substrates as novel biomarkers in prostate cancer.

15. SUBJECT TERMS
Histones, castration resistant prostate cancer, epigenetics, kinases.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU		

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INTRODUCTION

Epigenetic alterations leading to the silencing of tumor suppressor genes or activation of oncogenes are underlying causes for metastatic disease [1]. The reversible potential presented by these epigenetic modifications allows feasibility of targeted intervention. Recent studies have uncovered critical roles for histone tyrosine phosphorylation in genetic integrity, apoptosis, and oncogenesis [2]. The role of histone tyrosine phosphorylation in prostate cancer progression is unknown. The proposed project set out to identify and quantify novel histone tyrosine phosphorylation that may regulate the progression of castration resistant prostate cancer (CRPC). SILAC (Stable isotope labeling with amino acids in cell culture) based phosphoproteomic analysis will be utilized to identify and quantify novel histone tyrosine phosphorylations that may regulate the progression of castration resistant prostate cancer (CRPC). SILAC is a method for metabolic labeling that relies on incorporation of light (L) and heavy (H) amino acids that enable the mass spectrometer to differentiate between the peptides from two different samples.

BODY

Specific Aim1. Qualitative and quantitative differences in tyrosine phosphorylation of core histones isolated from androgen dependent and castration resistant cells

Prostate cancer cell lines (LAPC4 and LNCAP) were cultured in either in the presence or absence of synthetic androgen R1881. The cells were grown for at least seven passages in either heavy-isotope lysine [L-lysine:2HCl (U-13C6)] and arginine [L-arginine:HCl (U-13C6)] (castration resistant cells) or normal-isotope lysine and arginine (androgen-dependent cells). Each sample contained ~5 x 107 cells and LC-MS/MS was performed to confirm that the percentage of label incorporation was more than 98% of the protein. The cells were lysed in receptor lysis buffer (RLB) containing 25 mmol/L Tris (pH 7.5), 225 mmol/L NaCl, 1% Triton X-100, 1 mmol/L DTT, 10% glycerol, phosphatase inhibitors (10 mmol/L NaF, 1 mmol/L Na2VO4), and protease inhibitor mix (Roche). Total histones were purified using histone minipurification kit (Active motif) as per manufacturer's instructions, followed by enrichment on the immobilized metal affinity chromatography column and subjected to LC-MS/MS.

Results: After IMAC enrichment, histones were found to be modified at a number of residues including acetylation, ubiquitination and phosphorylation. Mass spectrometry data is shown below for some of the histone modifications that displayed significant changes (Fig 1-5). The role of these modifications in prostate cancer progression has not been studied earlier.

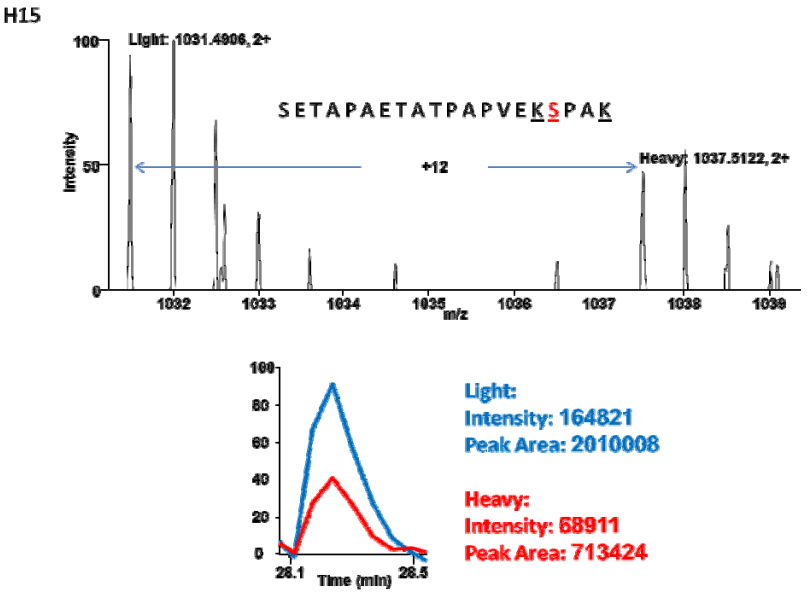


Fig 1: LC/MS analyses of the tryptic digest reveals H1 phosphorylated at serine 18 is significantly decreased in cells growing under androgen depleted conditions. This site may be phosphorylated by CDK1, but its relevance in prostate cancer pathology is not known.

Fig 2: SILAC based LC/MS analyses of the tryptic digest reveals H1.4 isoform phosphorylated at threonine 18 is significantly decreased in cells growing under androgen depleted conditions. This site may be phosphorylated by CDK1, but its relevance in prostate cancer pathology is also not known.

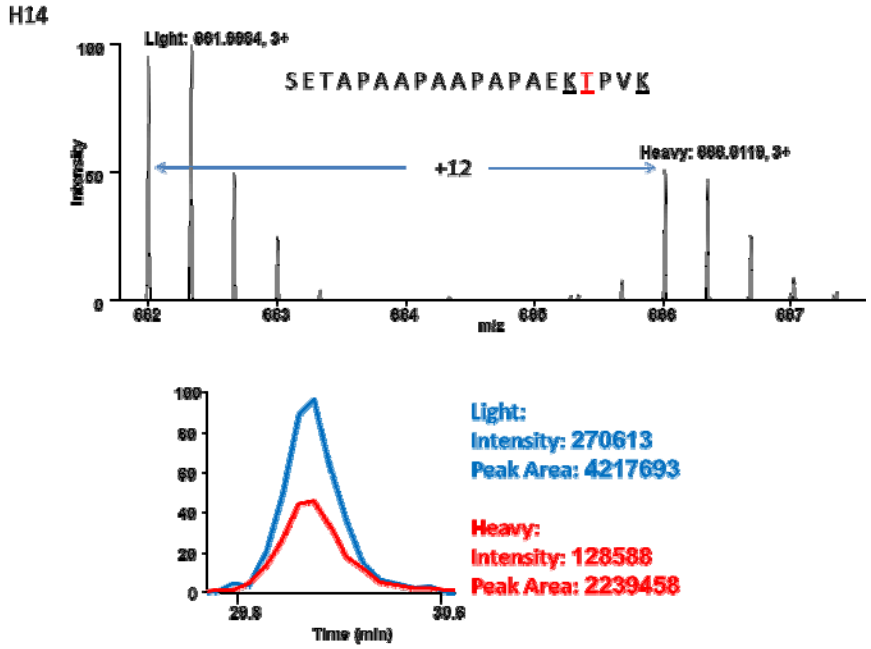
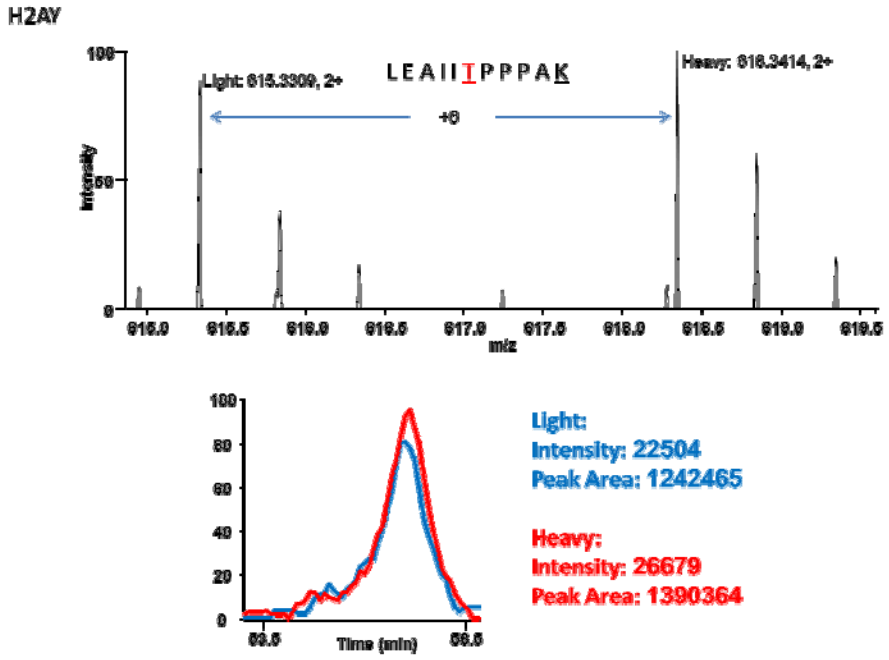


Fig 3: SILAC based LC/MS analyses of the tryptic digest revels that phosphorylation of Histone H2AY at threonine 129 is marginally increased in androgen depleted conditions. This site was found to be modified in earlier studies [3]. But its modification has not been investigated in prostate cancer.



Since lysine modifications on histones represent some of the other major epigenetic modifications deregulated in cancers, we analyzed the lysine modifications in histone preparations from both prostate cancer cell lines. Quantitative analysis revealed that the lysines were found to be modified in both androgen dependent and castration resistant cells to the same extent in majority of the histones. No changes were observed in Histone H4 at lysine 8 and lysine 12 acetylation under the two conditions (**Fig 4A**), except the lysine 5 in histone H2B, which was higher in the presence of androgen (**Fig 4B**). This residue may be modified by the p300/CBP histone acetyl transferases which are known to acetylate multiple lysine residues in the amino terminal tail of histone H2B (Lys5, 12, 15, and 20) at gene promoters during transcriptional activation [4, 5].

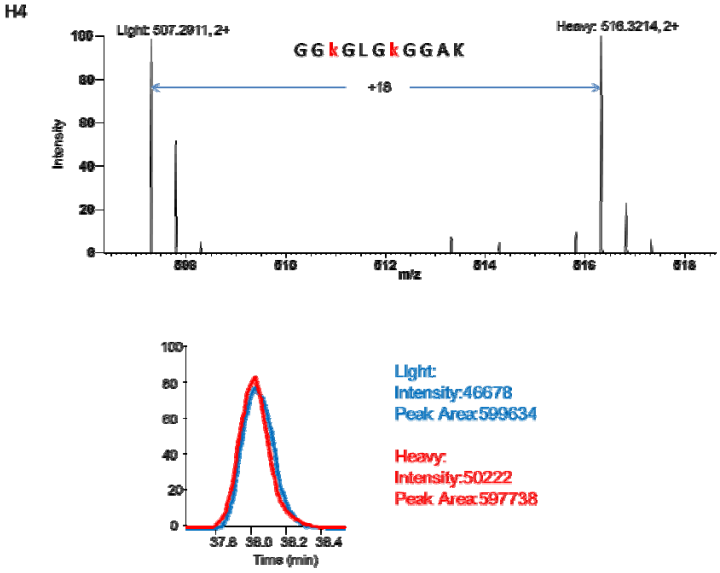


Fig 4A.

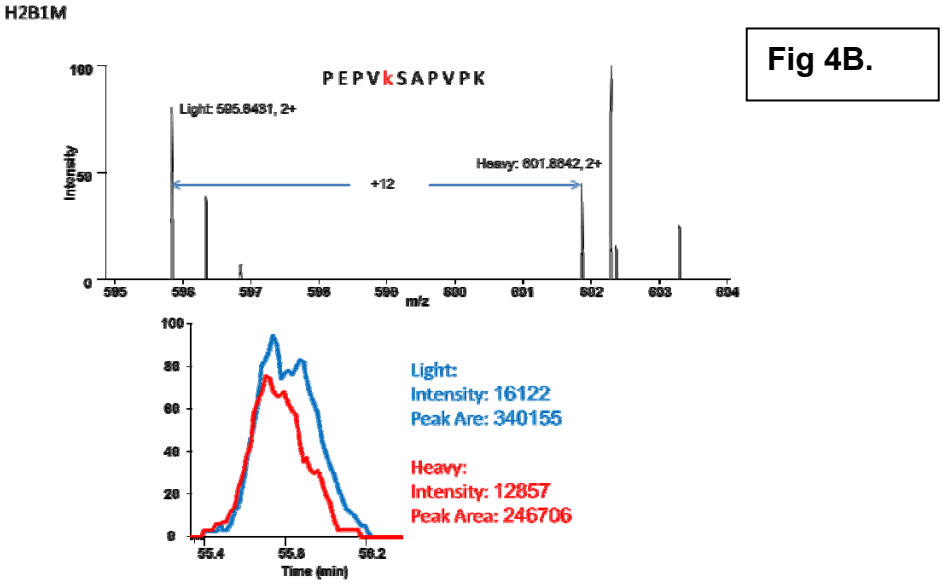
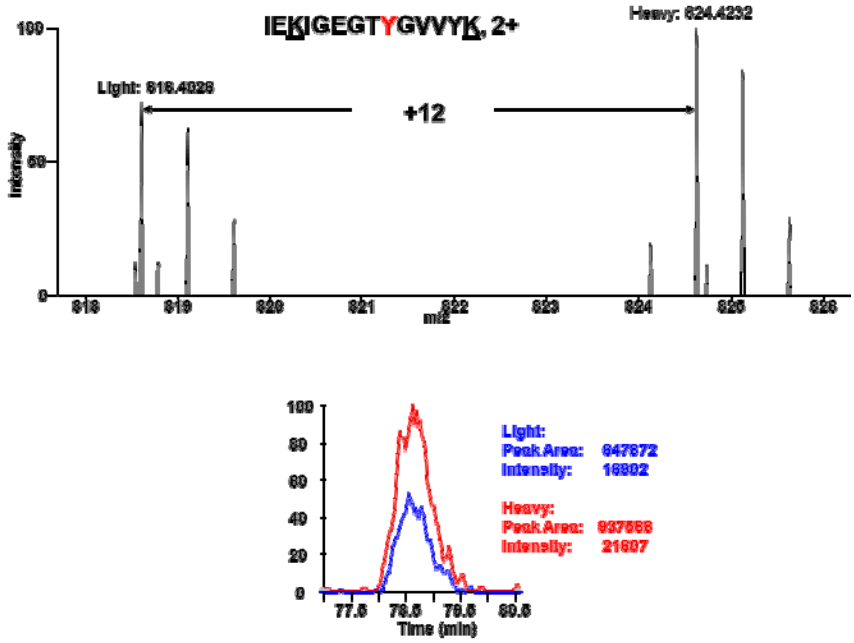


Fig 4B.

Specific Aim 2: Identification of the RTK pathways that regulate the histone tyrosine phosphorylation

The nonreceptor tyrosine kinases recruited along with AR at the promoter/enhancer elements may also modify histones by tyrosine phosphorylation to modulate expression of target genes that are proproliferative, confer survival advantage, and promote castration resistance [6]. In this subtask, prostate cancer cell lines were differentially labeled with either heavy-isotope lysine [L-lysine:2HCl (U-13C6)] and arginine [L-arginine:HCl (U-13C6)] in the absence of androgen were treated with tyrosine kinase inhibitors, Ack1 (5uM of AIM100) and for WEE1 (5 uM of MK1775) or DMSO as control. After confirming that 98% of the label was incorporated in a small fraction of the cells, the remaining cells were harvested, total histones were purified and quantitative mass was performed. Robust phosphorylation of ACK1 and WEE1 substrate, CDK1, were found in the cells growing in the absence of androgen, suggesting that these pathways are upregulated in prostate cancer cells and these cells are likely adapting to androgen depletion by upregulating ACK1 and the essential cell cycle kinase WEE1. There were around 10 phosphotyrosine (pY) sites on other non-histone proteins, such as in CDC2, CDK1/3, YES and PRP4.

Fig 5: The LC-MS/MS spectra for CDK1 is shown below.



Key Research Accomplishments

1. Quantitative LC-MS/MS analysis of epigenetic modifications in differentially labeled prostate cancer cell lines.

2. Identification of ACK1 and WEE1 kinase activity as being significantly upregulated in androgen-independent prostate cancers.

3. Future studies could explore CDK1 phosphorylation as novel biomarkers in prostate cancer.

Reportable Outcomes
Since these studies were exploratory there are no reportable outcomes yet. As the results suggest that some histone modifications are altered during disease progression, this will be used as preliminary data and will be investigated further when future funding is obtained.

Conclusions
Histones serine and threonine phosphorylation were abundant both in the presence and absence of androgen, while tyrosine phosphorylation could not be detected by mass-spectrometry analysis in two different prostate cancer derived cell lines LNCAP and LAPC4. Western blotting analysis with phosphotyrosine antibodies however revealed the presence of tyrosine phosphorylated histones, suggesting that this modification may not be amenable for mass spectrometry. Specific immunoblotting with anti-pY37-H2B antibodies' revealed the presence of tyrosine 37 phosphorylated H2B in LNCAP cells. This phosphorylation is mediated by WEE1 kinase.

WEE1 is also the kinase for cyclin-depndent kinase CDK1. Treatment with WEE1 inhibitor, MK1775, revealed the loss of CDK1 phosphorylation in LAPC4 prostate cell line suggesting that this pathway is active in prostate cancer cells. ACK1 and WEE1 were found to be some of the major pathways upregulated in prostate cancer cells upon androgen deprivation. Thus while mass spectrometry is a highly quantitative technique and sheds light on changes in other histone modifications, except for tyrosine which due to their low abundance and labile nature, are difficult to detect and quantitate. This problem can however be overcome by generating phospho-specific antibodies' and exploring the role of this modification in progression of prostate cancer to androgen independence.

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